

Q1 This application is a continuation of U.S. Application No. 09/501,708 filed February 10, 2000, which claims the benefit of U.S. Provisional Application Numbers 60/119,571 filed 2/10/1999, now abandoned, and 60/139,172 filed 6/15/99, now abandoned, all of which are hereby incorporated herein by reference in their entireties.

Please delete the BRIEF DESCRIPTION OF THE FIGURES section and replace it with the following replacement BRIEF DESCRIPTION OF THE FIGURES section.

FIG. 1A shows the sequence of a polynucleotide (SEQ ID NO: 1) which encodes human β -secretase translation product shown in FIG. 2A.

FIG. 1B shows the polynucleotide of FIG. 1A, including putative 5'- and 3'- untranslated regions (SEQ ID NO: 44).

FIG. 2A shows the amino acid sequence (SEQ ID NO: 2)[1-501] of the predicted translation product of the open reading frame of the polynucleotide sequence shown in FIGS. 1A and 1B.

Q2 FIG. 2B shows the amino acid sequence of an active fragment of human β -secretase (SEQ ID NO: 43)[46-501].

FIG. 3A shows the translation product that encodes an active fragment of human β -secretase, 452stop, (amino acids 1-452 with reference to SEQ ID NO: 2; SEQ ID NO: 59) including a FLAG-epitope tag (underlined; SEQ ID NO: 45) at the C-terminus.

FIG. 3B shows the amino acid sequence of a fragment of human β -secretase (amino acids 46-452 (SEQ ID NO: 58) with reference to SEQ ID NO: 2; including a FLAG-epitope tag (underlined; SEQ ID NO: 45) at the C-terminus.

FIG. 4 shows an elution profile of recombinant β -secretase eluted from a gel filtration column.

FIG. 5 shows the full length amino acid sequence of β -secretase 1-501 (SEQ ID NO: 2), including the ORF which encodes it (SEQ ID NO: 1), with certain features indicated, such as "active-D" sites indicating the aspartic acid active catalytic sites, a transmembrane region commencing at position 453, as well as leader ("Signal") sequence (residues 1-22; SEQ ID NO: 46) and putative pro region (residues 23-45; SEQ ID NO: 47) and where the polynucleotide region corresponding the proenzyme region corresponding to amino acids 46-501 (SEQ ID NO: 43)(nt 135-1503) is shown as SEQ ID NO: 44.

FIGS. 6A and 6B show images of silver-stained SDS-PAGE gels on which purified β -secretase-containing fractions were run under reducing (6A) and non-reducing (6B) conditions.

FIG. 7 shows a silver-stained SDS-PAGE of β -secretase purified from heterologous 293T cells expressing the recombinant enzyme.

FIG. 8 shows a silver-stained SDS-PAGE of β -secretase purified from heterologous Cos A2 cells expressing the recombinant enzyme.

FIG. 9 shows a scheme in which primers derived from the polynucleotide (SEQ ID NO. 76 encoding N-terminus of purified naturally occurring β -secretase (SEQ ID NO. 77) were used to PCR-clone additional portions of the molecule, such as fragment SEQ ID NO. 79 encoding by nucleic acid SEQ ID NO. 98, as illustrated.

FIG. 10 shows an alignment of the amino acid sequence of human β -secretase ("Human Impain.seq," 1-501, SEQ ID NO: 2) compared to ("pBS/mImpain H#3 cons") consensus mouse sequence: SEQ ID NO: 65.

Q² FIG. 11A shows the nucleotide sequence (SEQ ID NO: 80) of an insert used in preparing vector pCF.

FIG. 11B shows a linear schematic of pCEK.

FIG. 12 shows a schematic of pCEK.clone 27 used to transfect mammalian cells with β -secretase.

FIG. 13(A-E) shows the nucleotide sequence of pCEK clone 27 (SEQ ID NO: 48), with the OFR indicated by the amino acid sequence SEQ ID NO: 2.

FIG. 14A shows a nucleotide sequence inserted into parent vector pCDNA3 (SEQ ID NO: 80).

FIG. 14B shows a plot of β -secretase activity in cell lysates from COS cells transfected with vectors derived from clones encoding β -secretase.

FIGS. 15A shows an image of an SDS PAGE gel loaded with triplicate samples of the lysates made from heterologous cells transfected with mutant APP (751 wt) and β -galactosidase as control (lanes d) and from cells transfected with mutant APP (751 wt) and β -secretase (lanes f) where lanes a, b, and c show lysates from untreated cells, cells transfected with β -galactosidase alone and cells transfected with β -secretase alone, respectively, and lane e indicates markers.

FIG. 15B shows an image an image of an SDS PAGE gel loaded with triplicate samples of the lysates made from heterologous cells transfected with mutant APP (Swedish

mutation) and β -galactosidase as control (lanes c) and from cells transfected with mutant APP (Swedish mutation) and β -secretase (lanes e) where lanes a and b show lysates from cells transfected with β -galactosidase alone and cells transfected with β -secretase alone, and lane d indicates markers.

FIGS. 16A and 16B show Western blots of cell supernatants tested for presence or increase in soluble APP (sAPP).

FIGS. 17A and 17B show Western blots of α -cleaved APP substrate in co-expression cells.

Q² FIG. 18 shows A β (x-40) production in 293T cells cotransfected with APP and β -secretase.

FIG. 19A shows a schematic of an APP substrate fragment, and its use in conjunction with antibodies SW192 and 8E-192 in the assay.

FIG. 19B shows the β -secretase cleavage sites in the wild-type APP sequence (SEQ ID NO: 103) and Swedish APP sequence (SEQ ID NO: 104).

FIG. 20 shows a schematic of a second APP substrate fragment derived from APP 638, and its use in conjunction with antibodies SW192 and 8E-192 in the assay.

FIG. 21 shows a schematic of pohCK751 vector.

Please delete the paragraph at page 11, lines 4-5, and replace it with the following replacement paragraph.

Q³ SEQ ID NO: 51 is a peptide sequence cleavage site of APP (Swedish mutation).

Please delete the paragraph at page 11, line 8, and replace it with the following replacement paragraph.

Q⁴ SEQ ID NO: 54 is a peptide sequence cleavage site of APP (wild type) recognized by human β -secretase.

Please delete the paragraph at page 11, line 29, and replace it with the following replacement paragraph.

Q⁵ SEQ ID NO: 73 is P4-P4'staD \rightarrow V.

Please add the following paragraphs after the paragraph at page 11, line 26.

SEQ ID NO: 98 is a nucleic acid fragment (FIG. 9).

SEQ ID NO: 99 is the N terminal peptide sequence of β -secretase isolated from human brain, recombinant 293T cells and recombinant Cos A2 cells (Table 3).

SEQ ID NO: 100 is the N terminal peptide sequence of a form of β -secretase isolated from recombinant 293T cells.

Q6 SEQ ID NO: 101 is the N terminal peptide sequence of a form of β -secretase isolated from recombinant 293T cells.

SEQ ID NO: 102 is the N terminal peptide sequence of a form of β -secretase isolated from recombinant CosA2 cells.

SEQ ID NO: 103 is the β -secretase cleavage sites in the wild-type APP sequence.

SEQ ID NO: 104 is the β -secretase cleavage sites in the Swedish APP sequence.

Please delete the paragraph beginning at page 30, line 5, and replace it with the following replacement paragraph.

Q7 The full-length open reading frame (ORF) of human β -secretase is described above, and its sequence is shown in FIG. 2A as SEQ ID NO: 2. However, as mentioned above, a further discovery of the present invention indicates that the predominant form of the active, naturally occurring molecule is truncated at the N-terminus by about 45 amino acids. That is, the protein purified from natural sources was N-terminal sequenced according to methods known in the art (Argo Bioanalytica, Morris Plains, NJ,). The N-terminus yielded the following sequence: EGDEEPEEPGRRGSFVEMVDNLRG... (SEQ ID NO: 55). This corresponds to amino acids 46-69 of the ORF-derived putative sequence. Based on this observation and others described below, the N-terminus of an active, naturally occurring, predominant human brain form of the enzyme is amino acid 46, with respect to SEQ ID NO: 2. Further processing of the purified protein provided the sequence of an internal peptide: ISFAVSACHVHDEFR (SEQ ID NO: 56), which is amino terminal to the putative transmembrane domain, as defined by the ORF. These peptides were used to validate and provide reading frame information for the isolated clones described elsewhere in this application.

Please delete the paragraph beginning at page 31, line 8, (Table 3) and replace it with the following replacement paragraph (Table 3).

Table 3
N-terminal Sequences and Amounts of β -secretase Forms in Various Cell Types

Source	Est. Amount (pmoles)	N-terminus (Ref.: SEQ ID NO: 2)	Sequence
Human brain	1-2	46	<u>ETDEEPEEPGR...(SEQ ID NO: 99)</u>
Recombinant, 293T	~35 ~7 ~5	46 22 63	ETDEEPEEPGR...(SEQ ID NO: 99) TQHGIRL(P)LR...(SEQ ID NO: 100) MVDNLRGKS...(SEQ ID NO: 101)
Recombinant, CosA2	~4 ~3	46 58	ETDEEPEEPGR...(SEQ ID NO: 99) GSFVEMVDNL...(SEQ ID NO: 102)

Please delete the paragraph beginning at page 68, line 20, and replace it with the following replacement paragraph.

Recombinant proteins were generated with both the wild-type APP sequence (MBP-C125 wt) at the cleavage site (..Val-Lys-Met-Asp-Ala..) (SEQ ID NO: 54) or the "Swedish" double mutation (MBP-C125 sw) (..Val-Asn-Leu-Asp-Ala..) (SEQ ID NO: 51). As shown schematically in FIG. 19A, cleavage of the intact MBP-fusion protein results in the generation of a truncated amino-terminal fragment, with the new SW-192 Ab-positive epitope uncovered at the carboxy terminus. This amino-terminal fragment can be recognized on Western blots with the same Ab, or, quantitatively, using an anti-MBP capture-biotinylated SW-192 reporter sandwich format, as shown in FIG. 19A. Anti-MBP polyclonal antibodies were raised in rabbits (Josman Labs, Berkeley) by immunization with purified recombinantly expressed MBP (New England Biolabs). Antisera were affinity purified on a column of immobilized MBP. MBP-C125 SW and WT substrates were expressed in *E. coli*, then purified as described above.